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13. ABSTRACT *(Maximum 200 Words)*

We are developing gene therapy vectors derived from purified polyomavirus capsid proteins and genes assembled into chromatin. We hope to achieve selective targeting of the vectors by expressing on their surface, domains that bind receptors present on the surface of cancer cells. During the past year we have modified the polyomavirus VP1 capsid protein to contain sequences derived from urokinase plasminogen activator (uPA) responsible for binding to the urokinase plasminogen activator receptor (uPAr). Also, as a control, we have modified the VP1 protein with the FLAG epitope. The insertions of the uPA sequences have occurred at surface-exposed loops in the VP1 protein, defined by x-ray crystallographic analysis. These modified VP1 proteins have been expressed in E.coli and in insect cells. Methods for their purification have been developed, and their capacity to assemble into virion-like particles (VLPs) assessed by electron microscopy. While we have been able to assemble wild type VP1 expressed in either E.coli or insect cells into VLPs, only the modified VP1s expressed in insect cells form VLPs. This next year we pla to incorporate into them the chromatin-assembled DNAs and to assess their uptake by cells.

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Introduction:The most life-threatening aspect of cancer is its capacity to invade normal tissue and to establish new foci of tumor cells at distant sites. While there has been some progress in understanding some of the genetic and cellular mechanisms involved in the conversion of normal cells to metastatic tumor cells, little progress has been made in utilizing what has been learned of the molecular mechanisms of metastasis to reduce its morbidity and mortality. The objective of this work is to do that by developing novel gene therapy vectors selective for metastatic cells. Selectivity will rely upon metastatic cells expressing receptors to which the vectors can adsorb and upon the metastatic cells expressing signal transduction pathways which will activate vector gene expression.

Research Accomplished:

1. Construction of modified VP1 gene(s)

The polyomavirus major capsid protein VP1 will self-assemble to make virus-like particles (VLP) without involvement of other capsid proteins (Salunke DM. *et al.* 1986 Cell. 46(6): 895-904; An K *et al.*, 1999 J. Gen Virol. 80: 1009-16). VLP can package and transfer DNA into eukaryotic recipient cells. The relative simplicity of self-assembly makes polyomavirus an attractive candidate for a gene therapy vector.

VP1 is composed of 384 amino acids and its 3-dimensional structure has been reported (Soeda E *et al.* 1980). Four loops, BC, DE, EF and HI are exposed on the outside surface in capsomere structure and hence may not be involved in forming virus-like particles. We selected those 4 loops as target sites to insert foreign peptides.

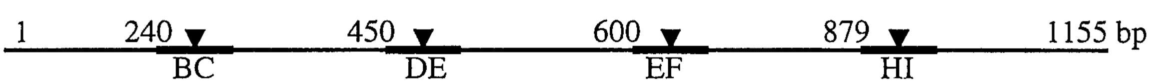
Structure of polyoma VP1 protein and sites of foreign peptide insertion

It has been shown that amino acid sequence of 13-32 of uPA is responsible for its binding to uPAR (Appella *et al.* 1987). Consequently, we have chosen to insert residues 10-34 of uPA into the VP1 protein so that it might interact with the uPAR receptor. The amino acid sequence of the uPA peptide(10-34) inserted into the VP1 gene is:

N C D C L N G G T C V S N K Y F S N I H W C N C P
10 15 20 25 30 34

It is possible that the uPA peptide inserted into VP1 protein might undergo conformational changes so that its binding capacity is lost. In that case, there will no specific interactions between virus-like particle and cancer cells and hence, no specific targeting. In such an event, a peptide that has high affinity for uPAR might be a possible alternative to the uPA peptide. Goodson RJ *et al.*(1994) selected 15-mer peptides with high affinity for uPAR using random bacteriophage display. The selected peptides have two short conserved sequences, neither of which is found in uPA protein. One such peptide, present in clone 20, (AEPMPHSLNFSQYLWYT), showed a higher affinity for the uPA receptor than original uPA peptide. The IC_{50} of that peptide for the uPAR was 0.01uM while that of uPA[12-32 C19A] was 0.25 uM.

We also have inserted this 20 residue peptide into the loops of VP1. To help evaluate the effect of insert size on VP1 structure, a FLAG epitope was inserted in the HI loop of VP1. Self-assembly of VP1:FLAG protein will be compared with those of other proteins which containing uPA peptide or clone 20 peptide.



VP1 protein: (▼ : Site of foreign peptide insertion)

2. Expression and Purification of VP1 protein(s) in *E. coli*

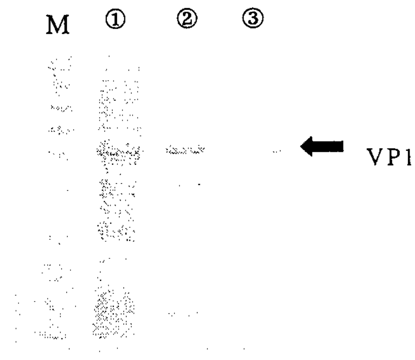
To insert the uPA peptide or clone 20 peptide sequence into the VP1 gene, PCR-based cloning was employed and the modified genes sequenced. Modified VP1 genes made from PCR were cloned into the pET-15b vector and expression of protein was investigated in BL21 LysS cells. Protein expression was confirmed by SDS-PAGE and western-blot analysis using anti-VP1 antibody.

We have applied the method for purifying expressed VP1 protein developed by Leavitt A. *et al.* (1985), to purify modified VP1 proteins. The major purification steps are as follows:

Purification of wild type and Modified VP1 proteins

Culture of bacterial cells
↓
Induction(0.2mM IPTG, 6hr at 28°C)
↓
harvest and sonication(supernatant:①)
↓
Polymin P precipitation(0.35% (w/v))
↓
Extract w/ high salt & $(NH_4)_2SO_4$ precipitation
↓
Resuspend pellet with buffer A(50mM NaCl)
↓
DEAE column(flow through: ②)
↓
Phosphocellulose column
(elute with 1M NaCl: ③)

Adopted from procedure by Leviatt *et al.* (8)
Modified proteins can be purified by same procedure.



SDS-PAGE of VP1 purification

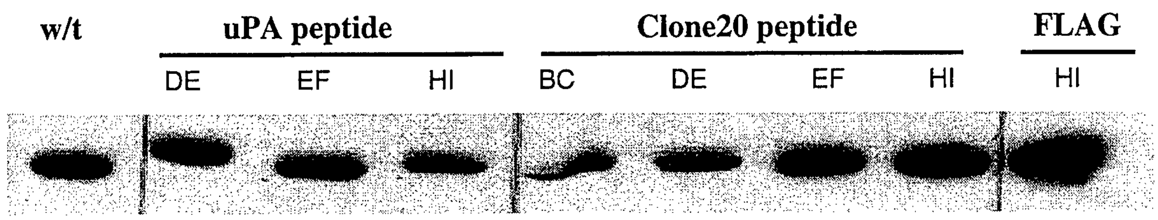
3. Self-assembly of VP1 protein(s) *in vitro*

Purified VP1 proteins undergo self-assembly in high ionic condition to make virus-like particles (VLPs). Once VLPs are formed, they can be stabilized in low ionic strength buffers (100mM NaCl) with calcium ions (Salunke DM *et al.* 1986). Wild type and modified VP1 proteins expressed in *E. coli* were incubated in buffer containing Tris 20 mM (pH = 7.3), ammonium sulfate (0.75 M), 5% glycerol and 1mM calcium chloride to allow self-assembly. Under transmission electron microscopy, assembled capsid-like particles look like native virus (Salunke DM *et al.* 1986, Forstova *et al.*, 1995).

We found with the E.coli expressed proteins, that only wild type VP1 protein self-assembles to make VLP, for the modified VP1 proteins undergo precipitation in the buffer and conditions used for self-assembly. To promote self-assembly of modified VP1 protein, various changes of assembly buffer were tried including addition of detergent, pH change (6.3-8.3) and concentration of calcium chloride. However none of these modifications were successful. Consequently, as an alternative, we turned to baculovirus expression of the VP1 proteins in insect cells, which have also been reported to support self-assembly of VP1 protein.

4. Cloning of wild type and modified VP1 gene(s) into baculovirus and protein expression:

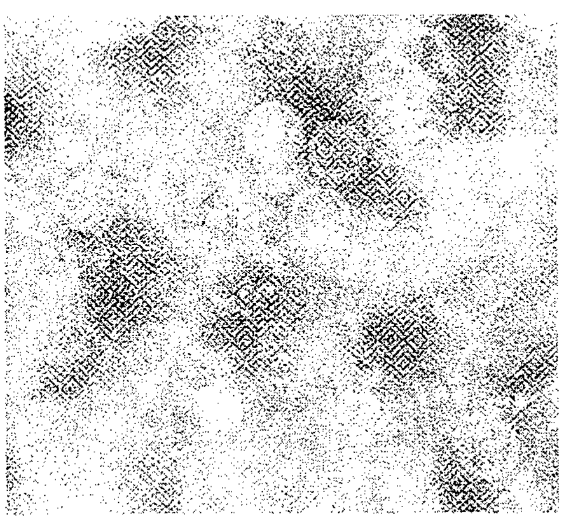
Wild type and modified VP1 gene(s) were cloned into the pFastBac expression plasmid and transformed into DH10Bac competent cells containing Bacmid plasmid DNA. Recombinant bacmids containing wild type or modified VP1 gene(s) were purified and transfected into Hi-5 insect cells to obtain recombinant baculovirus.These were amplified to obtain high titer virus stocks. To produce VLP, confluent monolayer of Hi-5 cells were infected with recombinant baculoviruses and incubated for 4 days. Protein production was evaluated by western blot analysis.



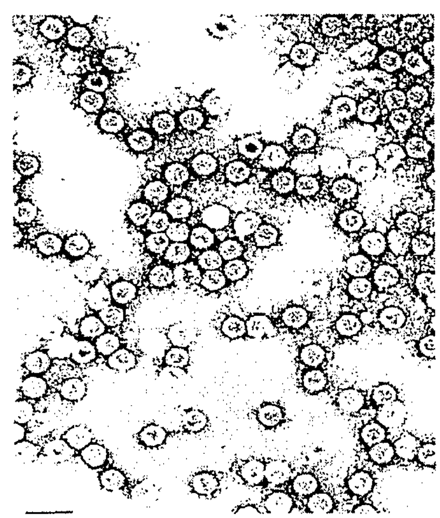
5. Purification of VLP and transmission electron microscopy

After harvesting the infected cells by low speed centrifugation, Hi-5 cells were disrupted by brief sonication and the lysate was centrifuged at 10.000g for 30minutes, with the supernatant being saved. The pellet was re-extracted by sonication and centrifuged same as above. The supernatant was saved and combined with the original supernatant. VLPs were concentrated through 2ml of a 20% sucrose shelf and centrifuged at 35,000rpm for 120 minutes in a Beckman SW40 rotor. The pellet containing the partially purified VLPs was resuspended in small volume of buffer and layered on top of a preset cesium chloride gradient (1.35g/ml-1.23g/ml) and centrifuged at 33,000rpm for 15 hours in a SW40 rotor and then fractions were collected. The fraction containing VLP was dialyzed and used for transmission electron microscopy. Unlike the modified VP1 proteins produced in *E.coli*, modified VP1 proteins produced in insect cells could self-assemble to make VLPs.

Electron microscopy image of VLPs



VLPs made from insect cell expressed VP1



Native polyoma virus

Key research Accomplishments to date:

1. We have expressed and purified polyomavirus VP1 proteins containing inserts derived from sequences that bind uPAR.

2. We have assembled virus like particles (VLPs) from these modified proteins.

Reportable Outcomes:

1.Development of clones that express chimeric VP1 proteins.

2.Development of procedures for the purification and self-assembly of modified VP1 proteins

3.Progress toward completion of a PhD degree by Mr Young-Shin, graduate student.

Conclusion: We have essentially completed Technical Objectives 1 & 2 of the Statement of Work. This year we will focus upon Technical Objective #3. I am requesting a no-cost extension of the grant for one year to allow us to complete Technical Objective #4.

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